

Fungal Extracts Detected in Eosinophilic Chronic Rhinosinusitis Induced Cytokines From the Nasal Polyp Cells

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Objectives/Hypothesis: The role of fungi in chronic rhinosinusitis (CRS) is still controversial. The present study was conducted to detect and identify fungal species from the nasal polyp tissues of eosinophilic and noneosinophilic CRS, and to determine the role of fungal antigens in cytokine production.

Study Design: Prospective study.

Methods: Thirty-five specimens of nasal polyps were collected from patients with CRS and examined for fungus using culture, histology, and polymerase chain reaction analysis. The secretion of 14 cytokines stimulated by fungal extracts using dispersed nasal polyp cells (DNPCs) was determined by multiplex immunoassay.

Results: There was no microbiological growth (including fungus) in the cultures of homogenized nasal polyps. Furthermore, Grocott methanamine silver staining for all nasal polyps showed no fungal bodies. Sixteen of 35 samples of the nasal polyps showed amplification of fungal DNA. In none of the mucosa of the sphenoid sinus was fungal DNA detected. The number of eosinophils in the nasal polyps in which fungal DNA was detected was significantly higher than in the nasal polyps in which fungal DNA was not detected ($P < .01$). The extract of fungus enhanced the secretion of eosinophil-associated cytokines such as interleukin (IL)-5, IL-13, IL-17A, and RANTES (regulated on activation normal T-cell expressed and secreted), and proinflammatory cytokines such as IL-6, IL-8, tumor necrosis factor- α , and granulocyte-macrophage colony-stimulating factor from DNPCs.

Conclusions: The present study offers direct evidence supporting that fungal elements modify the inflammatory response in the nasal polyps of eosinophilic CRS.

Key Words: Fungus, nasal polyp, polymerase chain reaction, cytokine, eosinophil.

Level of Evidence: NA

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INTRODUCTION

Chronic rhinosinusitis (CRS) is considered to be a multifactorial disease within a heterogeneous group of diseases, with different underlying etiologies and pathophysiologies. CRS has been classified broadly as CRS with nasal polyps (CRSwNP), CRS without nasal polyps, and allergic fungal rhinosinusitis (AFRS). In particular, CRSwNP may have a complex pathogenesis and is thought to arise from multiple factors including allergy. Microorganisms have always been popularly suspected in the pathology of CRSwNP. There is recent evidence suggesting that 1) *Staphylococcus aureus* enterotoxins, 2) type I hypersensitivity to fungus, and 3) non-immunoglobulin E (IgE)-mediated hypersensitivity to fungus may play a role in the pathogenesis of eosinophilic inflammation.¹ However, the role of the microor-

ganisms, particularly fungal pathogens, in the etiology of CRS remains largely unknown.

The role of fungi in CRS is still controversial. Conflicting with the prevailing belief that fungi were responsible for CRS in a selected group of patients with distinct pathophysiology, Ponikau et al.² and Braun et al.³ observed that fungus is a ubiquitous intranasal presence, identified in close to 100% of both CRS patients and controls. The former group also detected fungi along with eosinophil and eosinophil-degraded products with mucus. Shin et al.⁴ exposed peripheral blood mononuclear cells to fungal antigens in vitro and reported increased interleukin (IL)-5 and IL-13 production in 89% of CRS patients but not in controls. These observations formed the basis of the “fungal hypothesis of CRS.” As further evidence, nasal mucus or tissue from CRS patients triggered eosinophil migration,⁵ and *Alternaria* fungus in particular can directly induce eosinophil degranulation mediated by protease-activated receptor (PAR) activation.⁶

However, other investigators reported the absence of a universal hyper-responsiveness to fungal antigens in CRS patients.^{7,8} Furthermore, a multicenter, randomized clinical trial of topical antifungal agents for CRS eventually failed to show any evidence of efficacy,⁹ and a meta-analysis did not support the routine use of topical

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TABLE I.
Primers Used in the Study.

Primer	5' → 3'
ITS-IF	GTC GTA ACA AGG TTA ACC TGC GG
ITS-4R	TCC TCC GCT TAT TGA TAT GC
NL-1	GCA TAT CAA TAA GCG GAG GAA AAG
NL-4	GGT CCG TGT TTC AAG ACG G
FF-2	GGT TCT ATT TTG TTG GTT TCT A
FR-1	CTC TCA ATC TGT CAA TCC TTA TT

antifungals for CRS.¹⁰ Thus, the precise roles of fungi in the etiopathology of CRS remain unknown.

The present study was conducted to detect and identify fungal species from the nasal polyp tissues of eosinophilic and noneosinophilic CRS using Grocott methanamine silver staining and polymerase chain reaction (PCR) methods. Moreover, the effects of fungal extracts identified in the nasal polyps were examined by the ex vivo cellular responses of dispersed nasal polyp cells (DNPCs).

MATERIALS AND METHODS

Patients

Thirty-five patients with CRS with nasal polyps (21 males and 14 females, ranging in age from 23–77 years, mean age of 49 years) were consecutively recruited from the Department of Otorhinolaryngology of Juntendo University Hospital from April 2011 to March 2012. CRS with nasal polyps was diagnosed based on the criteria of the European position paper.¹¹ None of the patients was treated with antibiotics, systemic or topical corticosteroids, or other immune-modulating drugs for at least 1 month before the surgery. Subjects with AFRS were excluded from the present study. The criteria of AFRS of two positive findings, 1) specific IgE antibodies against fungi, and 2) the presence of fungi in the sinus effusion using Grocott methanamine cytological silver staining or microbiological examination. Serum fungus-specific IgE concentrations against *Alternaria*, *Aspergillus*, *Candida*, *Penicillium*, *Mucor*, *Cladosporium*, and *Pityrosporum* were measured. Patients with CRSwNP associated with current signs of purulent nasal discharge, chronic obstructive pulmonary disease, diffuse panbronchiolitis, fungal sinus disease, congenital mucociliary disease, or cystic fibrosis were excluded from this study. The control group consisted of 15 patients with pituitary tumor surgery (four males and 11 females, age range from 36 to 73 years, mean age of 55 years). The study was approved by the ethics committee of the Juntendo University Faculty of Medicine.

Sampling of Tissue and Pretreatment

Surgically removed human nasal polyps located in the middle meatus were obtained from the patients with CRSwNP, and the mucosa of the sphenoid sinus as a control were procured from patients with pituitary tumor. They were treated

with 70% ethanol and physiologic saline to eliminate microorganisms outside of the nasal polyps. The samples were placed immediately in a 50% glycerol, then transferred to a –80°C freezer for storage until DNA extraction could be performed. At the same time, some of these samples were fixed in 10% formalin, embedded in paraffin wax, processed routinely, and stained with hematoxylin-eosin. According to our previous studies,^{12,13} the eosinophilic and noneosinophilic groups were defined as eosinophil counts of the nasal polyps of more and less 100/microscopic field (magnification ×400) using three fields, respectively. Eosinophils were quantified in the foci of the densest cellular infiltrate. The total number of eosinophils present with a 10 × 10-mm reticulate present in the eyepiece was determined as the count per high-power field. The samples for the DNPCs were treated within 30 minutes postoperatively.

Culture and Histology of Fungi

After the nasal polyps were disrupted with glass beads and 100 µL of Tris-EDTA (10 mM Tris-Cl, 1 mM ethylenediaminetetraacetic acid pH 8.0) using a homogenizer (BioMasher) (Takara Bio Inc., Otsu, Japan), and 1 µL of homogenized polyp samples were spread onto 5% sheep blood agar, chocolate agar, Columbia anaerobic blood agar, Drigalski agar, and Sabouraud agar, and incubated for 2 weeks at 35°C. All specimens were stained with Grocott methanamine silver staining for confirmation of the existence of fungal organisms.

PCR of Fungi in the Nasal Polyps

DNA was extracted from the tissue of homogenized nasal polyps. PCR amplification of fungal specific ribosomal DNA was performed using ITS-1F and ITS-4R primer, the PCR product covering the end of the 18S rDNA gene to the start of 26S rDNA gene and NL-1 and NL-4 primer, the PCR product covering D1/D2 26S rDNA (Table I). A universal fungal primer set designed by the National Institute for Occupational Safety and Health, FF2 and FR1 primers specific for the amplification of 18S rDNA, was also used. Amplification was performed in a TaKaRa PCR Thermal Cycler Dice Gradient (Takara Bio Inc., Otsu, Japan) according to the manufacturer's specifications.

Species Identification

PCR products were purified by the High Pure PCR Product Purification Kit (Roche, Indianapolis, IN). These amplifications were sequenced using ITS1F, ITS4R, NL1, NL4, FF2, and FR1 primers by a Big Dye Terminator V3.1 Cycle sequencing kit and ABI sequence analyzer 3730×I (Applied Biosystems Inc., Carlsbad, CA). Species identification was determined by a BLAST search (DNA Data Bank of Japan, Mishima, Japan).

Cell Cultures and Multiplex Immunoassay

DNPCs were prepared from nasal polyps of eosinophilic CRSwNPs in five randomly selected patients by enzymatic digestion, as described by Okano et al.¹⁴ In flat-bottomed 24-well culture plates (Nunc, Roskilde, Denmark), 500 µL of 1 × 10⁶/mL DNPCs were stimulated with serial concentrations (2 and 200 µg/mL) of *Candida parapsilosis*, *Rhodotorula mucilaginosa*. The crude extracts of *C. parapsilosis* and *R. mucilaginosa* were provided by Teikyo University Japanese Society for Medical Mycology Research Center. After the incubation, freeze-dried fungi were dissolved and sonicated in phosphate-buffered saline (PBS) with 0.2% NaN₃. Afterward, the extract was subjected to sterilizing filtration and lyophilization. The antigen extracts of fungus were dissolved with PBS (Sigma-Aldrich Inc., St. Louis, MO). As a control, DNPCs were cultured without antigen stimulation.

TABLE II.
Clinical Characteristics of Patients and Identified Fungal Species.

Patient	Sex/Age, yr	Blood Eosinophils	Blood IgE	RAST Fungus	Asthma	Eosinophilic Mucin	Tissue Eosinophils	Identified Fungal Species
1	M/47	420	38	—	+	+	58	<i>Candida parapsilosis</i>
2	M/36	508	332	+	+	+	148	<i>Candida parapsilosis</i>
3	F/74	301	65	—	+	—	111	<i>Rhodotorula mucilaginosa</i>
4	F/35	644	77	—	+	—	410	<i>Rhodotorula mucilaginosa</i>
5	M/45	328	162	—	+	—	184	<i>Rhodotorula mucilaginosa</i>
6	M/67	809	301	—	+	—	275	<i>Malassezia restricta</i>
7	M/39	462	118	—	+	—	164	<i>Aspergillus</i> s/o
8	F/46	381	1,990	—	+	—	194	<i>Candida parapsilosis</i>
9	M/42	418	86	—	+	—	291	<i>Aspergillus gracillus</i>
10	M/44	675	51	—	+	—	650	<i>Candida parapsilosis</i>
11	M/49	428	517	—	—	—	198	<i>Rhodotorula mucilaginosa</i>
12	F/49	731	430	+	—	—	189	<i>Candida parapsilosis</i>
13	M/39	412	46	—	—	—	341	<i>Candida parapsilosis</i>
14	F/51	353	89	—	—	—	184	<i>Candida glabrata</i>
15	F/62	192	38	—	—	—	176	<i>Candida tropicalis</i>
16	F/55	280	1,882	+	—	—	266	<i>Candida parapsilosis</i>
17	M/49	420	104	—	+	+	232	None
18	M/45	328	162	—	+	—	124	None
19	F/60	468	191	—	+	—	158	None
20	M/32	538	25	—	+	—	175	None
21	M/66	271	83	—	—	—	18	None
22	F/34	795	5	—	—	—	91	None
23	F/56	99	24	—	—	—	14	None
24	M/25	152	118	—	—	—	12	None
25	F/60	1,372	191	—	—	+	158	None
26	F/58	413	65	—	—	+	186	None
27	M/63	567	515	+	—	+	58	None
28	F/57	413	104	—	—	—	153	None
29	M/42	246	250	—	—	—	111	None
30	M/44	292	91	—	—	—	128	None
31	F/52	270	287	—	—	—	36	None
32	M/76	7	11	—	—	—	27	None
33	F/63	384	528	+	—	—	146	None
34	F/34	30	1,409	+	—	—	148	None
35	M/43	150	500	+	—	—	66	None

F = female; IgE = immunoglobulin E; M = male; RAST = radioallergosorbent test.

The culture supernatant was collected after 72 hours and stored at -80°C , after which the levels of IL-4, IL-5, IL-6, IL-8, IL-13, IL-17A, IL-23, IL-25, IL-33, eotaxin, RANTES (regulated on activation normal T-cell expressed and secreted), tumor necrosis factor (TNF)- α , interferon (IFN)- γ , and granulocyte-macrophage colony-stimulating factor (GM-CSF) were measured by Bio-Plex Suspension Array System (Bio-Rad Laboratories, Inc., Hercules, CA). Data were expressed as the fold change relative to that without stimulus of five cell cultures. Viability was assessed by the exclusion of trypan blue stain.

Statistical Analyses

Values are given as means \pm standard errors for the multiplex immunoassay. Differences between the values were determined using the Student *t* test. Tukey's hinge was used in the

comparison of eosinophils within nasal polyps. *P* values $<.05$ were considered significant. All analyses were conducted using Statmate IV for windows (ATMS Co., Ltd., Tokyo, Japan).

RESULTS

Culture and Histology of Fungi

There was no microbiological growth (including fungus) in the culture media of the homogenized nasal polyps. Furthermore, Grocott methanamine silver staining for the all nasal polyps showed no fungal bodies.

Detection and Identification of Fungal DNA

Sixteen of 35 samples of the nasal polyps showed amplification of fungal DNA. Using the D1/D2 domain of

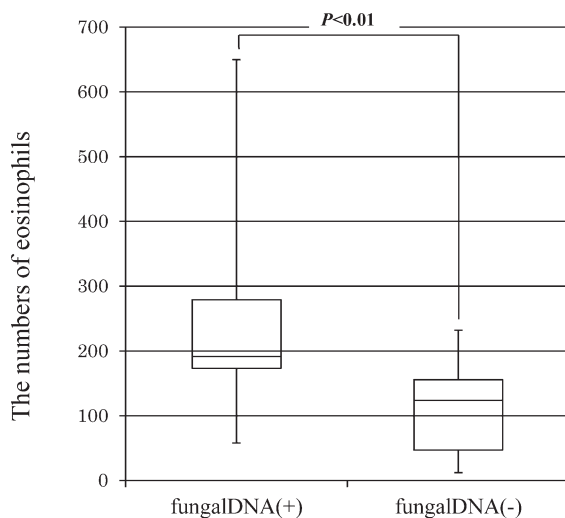


Fig. 1. Comparison of the numbers of tissue eosinophils with nasal polyps between positive samples and those negative for fungal DNA. The number of eosinophils in three fields with cell clusters were counted using light microscopy ($\times 400$ magnification). The box and whisker plots show the median and the interquartile range of the number. The whiskers extend to the maximum and the minimum data points.

the large subunit (26S) ribosomal DNA primer, NL1 / NL4, we found that 16 cellular tissues of the homogenized nasal polyps (100%) were positive for fungal DNA, whereas using the 18S rDNA primer, ITS1F/ITS4R amplified sequences four of the 16 (25%) were positive. On the other hand, using the universal fungal primer set, FF2 and FR1, no fungal DNA was detected. Incidentally, in none of the mucosa of the sphenoid sinus was fungal DNA detected. The amplification products obtained from 16 patients were sequenced to identify the detected species in the tissue of the homogenized nasal polyps, such as *C parapsilosis*, *R mucilaginosa*, and *Aspergillus* sp. Table II summarizes the data for all patients. The average number of eosinophils within the nasal polyps in which fungal DNA was detected was significantly higher than that in the absence of fungal DNA ($P < .01$, Fig. 1).

Cytokine Secretion Stimulated by Fungal Extracts

To determine the biological and immunological roles of the identified fungi in CRSwNP in the present study, five samples of DNPCs were stimulated by extracts of *C parapsilosis* and *R mucilaginosa*. The expression levels of each cytokine and chemokine are summarized and compared with those of the unstimulated group in Figure 2). The secretion levels of the cytokines were set to the maximal level of each standard curve because the levels were above the maximal levels of detection.

C parapsilosis stimulation of DNPCs significantly induced the secretion of IL-5, IL-6, IL-8, IL-13, RANTES, TNF- α , and GM-CSF. The most remarkable upregulation was observed in IL-6 (approximately 100-fold) and IL-8 (approximately 60-fold), followed by IL-5, TNF- α , RANTES, IL-13, and GM-CSF. IL-4, IL-17A,

IL-23, and eotaxin showed no significant increase from stimulation by *C parapsilosis* extracts. The expression of IL-25, IL-33 and IFN- γ showed a decreasing trend. No significant differences were seen in the responses between 2 and 200 $\mu\text{g/mL}$ of any cytokines.

The stimulation of DNPCs by *R mucilaginosa* resulted in a significant induction of IL-5, IL-8, IL-13, IL-17A, RANTES, TNF- α , and GM-CSF. Similar to *C parapsilosis*, *R mucilaginosa* most strongly upregulated the production of both IL-6 and IL-8, which was followed by IL-5, TNF- α , RANTES, IL-13, IL-17A, and GM-CSF. IL-4, IL-23, IL-25, IFN- γ , and eotaxin showed no significant production with stimulation of *R mucilaginosa* extract. On the other hand, *R mucilaginosa* inhibited IL-33 production. There was no significant difference in the cytokine releases induced by 2 and 200 $\mu\text{g/mL}$.

DISCUSSION

In the present study, we found fungal DNA in the nasal polyps in spite of the lack of detection of fungus using histology and culture. A variety of studies agree that PCR is superior to both culture and Grocott methanamine silver staining for detecting fungal elements.¹⁵ Because the PCR studies showed the ubiquitous presence of fungi such as *Aspergillus*, *Penicillium*, *Cladosporium*, *Candida*, *Aureobasidium*, and *Alternaria* in the nose and paranasal sinuses of both CRS patients and healthy controls,^{16–18} it is unlikely that fungal species and fungal load play a role in disease development.¹⁹ Gosepath et al.²⁰ reported that fungal DNA was detected in all 27 CRS specimens with universal PCR primers. The differences in the detection rates of fungal DNA between Gosepath et al.'s report and our study may be due to the sampling method, surgical specimens, or environmental factors. On the other hand, Rao et al.²¹ reported that the proper design of PCR primers for fungi and the meticulous harvesting of nasal mucosa resulted in a low but significant incidence of fungi in CRS. The primer pairs NL1/NL4, which encode the D1/D2 domain of large subunit (26S) ribosomal DNA, have been widely used for the detection of fungal DNA and the identification of fungal species, as compared with two other primers, ITS1F/ITS4R and FF2/FR1.^{22,23} No PCR products could be obtained from normal mucosa as negative controls. Positive and negative controls were correctly amplified in every experiment. Therefore, the three PCR amplified systems used here were appropriately operated. The exact reason why the detection rates of three PCR systems showed different values is not known. The large amount of human DNA extracted from the nasal polyps might have inhibited several fungus-specific PCR products, because fungi share the same class of ribosomal RNA with humans. We detected PCR products derived from four species of fungi including *Candida*, *Rhodotorula*, *Malassezia*, and *Aspergillus* in the nasal polyps, which were never detected in the normal sinus mucosa. The *Rhodotorula* species are ubiquitous saprophytic yeast that can be recovered from many environmental sources. Previously reported as nonpathogenic, *Rhodotorula* species have emerged as opportunistic

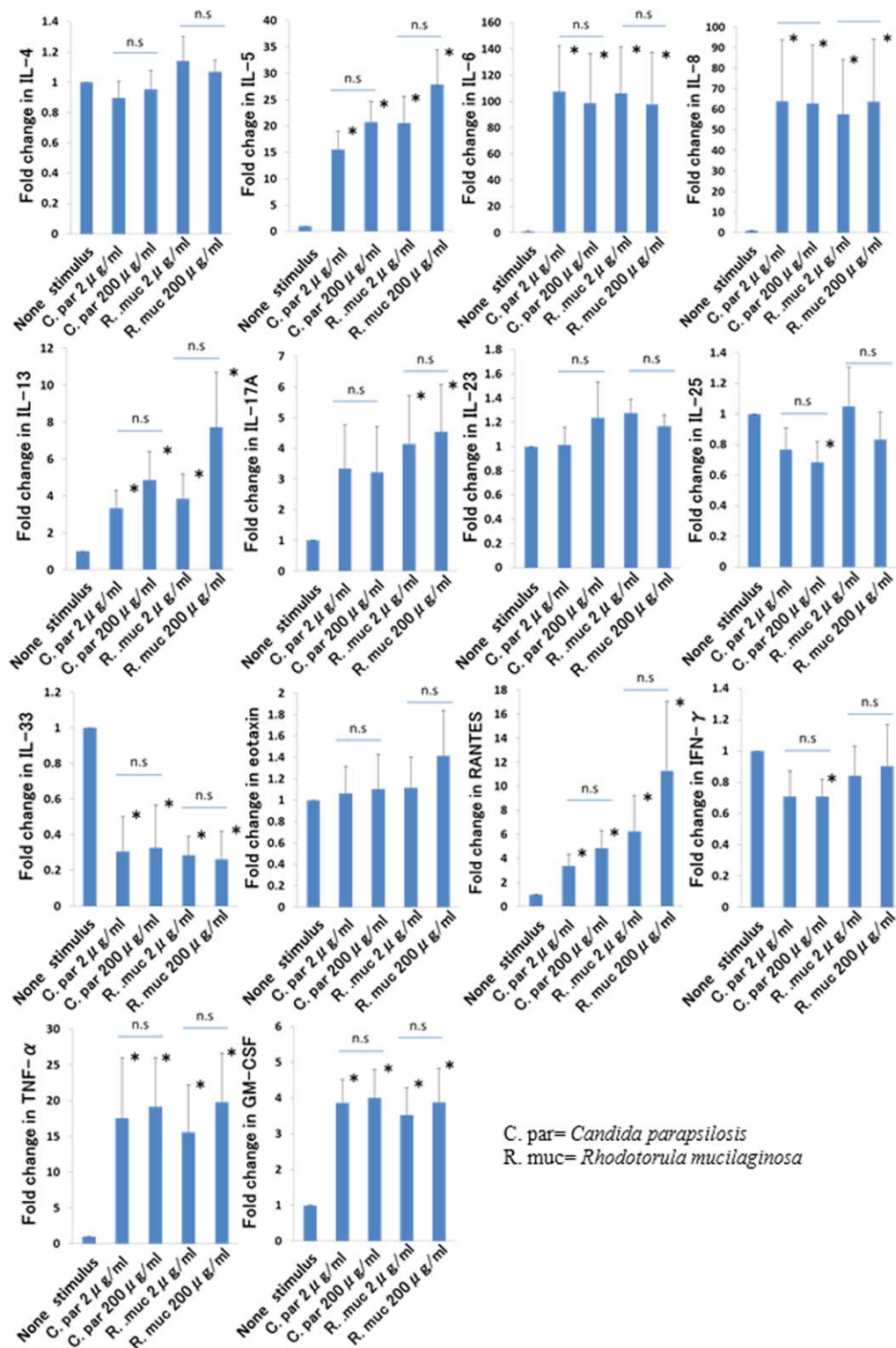


Fig. 2. Effect of extracts of *Candida parapsilosis* and *Rhodotorula mucilaginosa* on interleukin (IL)-4, IL-5, IL-6, IL-8, IL-13, IL-17A, IL-23, IL-25, IL-33, eotaxin, RANTES, interferon (IFN)- γ , tumor necrosis factor (TNF)- α , and granulocyte-macrophage colony-stimulating factor (GM-CSF) production by dispersed nasal polyp cells. Five hundred microliters of 1×10^6 /mL dispersed nasal polyp cells were collected after 72 hours, and the levels of the cytokines were determined by multiplex immunoassay. Data were expressed as the fold change relative to that without stimulus of five cell cultures (the means \pm standard errors). Significant differences (*) were considered if P values were $< .05$. RANTES = regulated on activation normal T-cell expressed and secreted.

pathogens with the ability to colonize and infect susceptible patients.²⁴ *Malassezia* is a monophyletic genus of fungi detected as a ubiquitous component of the human skin microbiome and is associated with a myriad of skin problems.²⁵ Although we attempted to observe the localization of the DNA of *Candida* and *Rhodotorula* using in situ hybridization in the nasal polyps, the experimental results failed to detect the fungal element, presumably due to the use of inappropriate probes. The fungal DNA would be identified within bacterial biofilms²⁶ and/or phagocytic vesicles of antigen-presenting cells such as macrophages and neutrophils in the nasal polyps.²⁷

The detection rate of fungal DNA was significantly elevated in the eosinophilic CRS as compared with non-eosinophilic CRS, suggesting that the presence of fungi is closely related with eosinophil accumulation. Eosinophils are known to be prominent in the reaction to parasitic infections. A concentration-dependent increase in eosinophil migration toward both CRS nasal mucin and CRS nasal tissue extract was augmented as compared with the mucin of healthy controls.⁵ Exposure of peripheral blood mononuclear cells to fungal antigen in vitro increased IL-5 and IL-13 production, whereas cells from normal controls did not respond.⁴ A component of *Alternaria* was shown to degranulate eosinophils from CRS patients by acting on PARs. Moreover, activation of nasal epithelial cells with fungi resulted in the upregulation of PAR2 and PAR3 mRNA.²⁸ These findings may lead to a hypothesis that fungi on the sinus mucosal surface induce the production of cytokines, which promote eosinophil migration through the epithelial cells and other constitutive cells of the nasal polyps. Although we did not employ quantitative assays of PCR products, it was considered that the patients with more fungal DNA would have significantly higher eosinophil counts.

The final series of experiments aimed to evaluate immunological aspects of the detected fungi in the induction of eosinophilic inflammation. The fungal extracts derived from *Candida* and *Rhodotorula*, which were detected from the nasal polyps, apparently and remarkably upregulated a Th1/Th2 and Th1Th2/Th17 cytokine profile, respectively, in the ex vivo models of the nasal polyp in the present study. It would be desirable to have stimulated the cytokine-producing cells from healthy sphenoid control tissue. However, it is well known that healthy tissue does not contain eosinophils and very minimal amounts of lymphocytes if any. Thus, no quantitative comparison would have been useful. Protease activity contained in fungi can activate epithelial cells via their PARs. Kaufman et al.²⁹ showed that the interaction of protease present in fungal extracts from the inferior nasal conchae of nonatopic subjects led to morphologic change, cell desquamation, and the induction of proinflammatory Th1 cytokines. In addition, PAR2 stimulation in CRS patients did not induce the release of eosinophil attracting cytokines like eotaxins or RANTES.³⁰ Recent investigations are focusing on the contribution of Th17 cells to the pathology of and resistance to fungi. Zelante et al.³¹ found that by affecting fungal clearance and by promoting chronic inflammation and tissue damage, Th17 responses at the mucosal sur-

face had a detrimental effect on the course of fungal infections. Our previous study revealed that the infiltration of cells positive for both CD4 and IL-17A (Th17 cells) showed a significant correlation with the numbers of eosinophils and mucosal remodeling in Japanese CRSwNP.¹²

Another underlying mechanism that might explain the fungus-mediated secretion of cytokines and chemokines, such as IL-6 and IL-8, from DNPCs may involve toll-like receptors (TLRs) expressed on the sinonasal mucosa. The recognition of various pathogen-molecular patterns by the TLRs, including mannan, *Candida* and CpG DNA induces a cascade of downstream signaling that provokes an inflammatory cytokine profile.³² A quantitative increase in TLR2 mRNA was seen in cystic fibrosis polyps as well as in CRS,^{33,34} whereas in some studies^{35,36} a decrease in mucosal TLR2 and TLR9 mRNA in samples from CRSwNP occurred. Despite inconsistencies in previous data, TLR signaling appears to play an important role in mediating host inflammation, with potential derangements contributing to the development of CRS.³⁷ Moreover, nucleotide-binding oligomerization domain-like receptors (NLRs) are newly discovered cytosolic receptors belonging to the pattern-recognition receptor family. NLR mRNA was found to be higher in nasal polyps than in normal nasal mucosa.³⁸ Further studies are required to elucidate the role of innate immunity in fungus-related CRS.

CONCLUSION

The present study offers direct evidence to support the notion that fungal elements modify the inflammatory responses in the nasal polyps of eosinophilic CRS, though the underlying mechanisms responsible for the eosinophil-based inflammation require further examination.

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